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Tryptophan Phosphorescence Spectroscopy Reveals That a Domain in the NAD(H)-binding Component (dI) of Transhydrogenase from *Rhodospirillum rubrum* Has an Extremely Rigid and Conformationally Homogeneous Protein Core*

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The characteristics of tryptophan phosphorescence from the NAD(H)-binding component (dI) component of *Rhodospirillum rubrum* transhydrogenase are described. This enzyme couples hydride transfer between NAD(H) and NADP(H) to proton translocation across a membrane and is only active as a dimer. Tryptophan phosphorescence spectroscopy is a sensitive technique for the detection of protein conformational changes and was used here to characterize dI under mechanistically relevant conditions. Our results indicate that the single tryptophan in dI, Trp-72, is embedded in a rigid, compact, and homogeneous protein matrix that efficiently suppresses collisional quenching processes and results in the longest triplet lifetime for Trp ever reported in a protein at ambient temperature (2.9 s). The protein matrix surrounding Trp-72 is extraordinarily rigid up to 50 °C. In all previous studies on Trp-containing proteins, changes in structure were reflected in a different triplet lifetime. In dI, the lifetime of Trp-72 phosphorescence was barely affected by protein dimerization, cofactor binding, complexation with the NADP(H)-binding component (dIII), or by the introduction of two amino acid substitutions at the hydride-transfer site. It is suggested that the rigidity and structural invariance of the protein domain (dI.1) housing this Trp residue are important to the mechanism of transhydrogenase: movement of dI.1 affects the width of a cleft which, in turn, regulates the positioning of bound nucleotides ready for hydride transfer. The unique protein core in dI may be a paradigm for the design of compact and stable *de novo* proteins.

Transhydrogenase couples hydride transfer between NAD(H) and NADP(H) to proton translocation across a biological membrane according to Equation 1.



The enzyme is found in the inner membrane of higher animal mitochondria and in the cytoplasmic membrane of many bac-

teria (1). Under physiological conditions, it is thought to utilize the proton electrochemical gradient to generate NADPH. Transhydrogenase comprises three components: dI¹ (~380 residues), which binds NAD⁺/NADH; dII (~400 residues), the membrane-embedded component harboring the proton translocation channel; and dIII (~200 residues), which binds NADP⁺/NADPH (Fig. 1). Transhydrogenase is a “dimer” of two dI-dII-dIII “monomers”, though the polypeptide composition varies in different species. One of the best characterized transhydrogenases is that from *Rhodospirillum rubrum*. Recombinant dI and dIII from this organism are stable proteins that bind their cognate nucleotides. Upon mixing, they readily form a dI₂-dIII₁ complex ($K_d \approx 25$ nM), which catalyzes rapid hydride transfer between bound nucleotides. Crystal structures are available for isolated dI (2, 3), dIII (4, 5), and for the complex (6).

In transhydrogenase, proton translocation is linked to the redox reaction by way of inter-domain conformational changes. Our current working model is that proton translocation through dII drives transhydrogenase between an “open” state, in which the nucleotide products are replaced with fresh substrates from the solvent, and an “occluded” state, in which the hydride transfer reaction takes place. The nature of the coupling reactions that link events during proton translocation with those at the hydride transfer site is not understood. Asymmetries in the dI₂-dIII₁ complex and half-site reactivity in the intact enzyme suggest that the conformations of the two monomeric units might alternate during turnover (1, 7). Experiments to investigate the conformation of dI and dIII under mechanistically relevant conditions should provide information on the coupling between the two components and between monomers within the dimer. In this report, we describe observations on the phosphorescence emission of a unique Trp residue at position 72 in *R. rubrum* dI. At micromolar concentrations, isolated dI from *R. rubrum* is a dimer: the K_d is unknown, but experiments suggest that the protein retains its dimeric character even at nanomolar concentrations (8). The dI protein comprises two domains, designated dI.1 and dI.2, which are separated by a deep cleft (Fig. 1). The x-ray structures reveal extensive contact areas between the dI.2 domains of the dimer polypeptides. Trp-72 is located on strand β d close to the center of domain dI.1. Its side chain is shielded from the solvent by

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¹ The abbreviations used are: dI, NAD(H)-binding component of transhydrogenase; dI.1 and dI.2, domains 1 and 2 of dI; dII, the membrane-spanning component of transhydrogenase; dIII, the NADP(H)-binding component of transhydrogenase; BW, bandwidth in nm of the 0,0 vibronic band at 1/2 peak height; $\lambda_{0,0}$, peak wavelength of the 0,0 vibrational band in nm; τ , phosphorescence lifetime; k_q , bimolecular quenching constant.

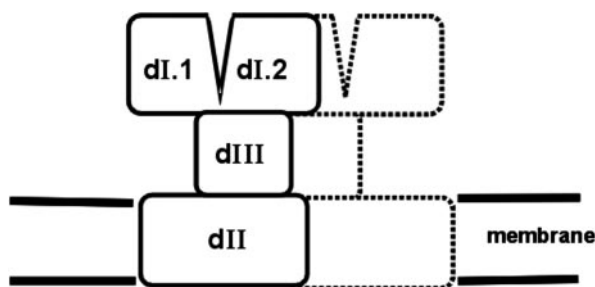


FIG. 1. The global organization of the components of transhydrogenase. The enzyme is shown as a dimer of two dI-dII-dIII monomers. One monomer is displayed with solid lines, the other with dotted lines. The division of dI into two domains (dI.1 and dI.2) with an intervening cleft is illustrated. The two nucleotide-binding components (dI and dIII) protrude from the membrane to the cytoplasmic side in bacteria and the matrix side in mitochondria.

amino acid residues on helices $\alpha 1$, $\alpha 11$, and loop $\beta \alpha$ - $\alpha 1$ (Fig. 2). Trp fluorescence spectroscopy was used to study the interaction of dimeric dI with substrates (9). There was no evidence for co-operative binding of NADH.

The phosphorescence emission of Trp in proteins has recently emerged as a complementary monitor of local protein structure, standing out for its remarkable sensitivity to the nature, dynamic make-up, and conformational uniformity of the protein environment. The sharp vibrational structure of the phosphorescence spectrum gives detailed information on the polarity and conformational homogeneity of the Trp environment (10, 11). In the absence of internal quenching by proximal cysteine, cystine, tyrosine, or histidine residues, the phosphorescence lifetime of proteins in fluid solutions provides a direct probe of the local flexibility of the protein matrix. The lifetime ranges from sub-milliseconds, when the Trp side chain is in unstructured fluid regions of the polypeptide, to seconds for completely buried and immobilized sites (12, 13). Additional information on structural flexibility can be derived from the accessibility of small solutes, such as oxygen and acrylamide, that migrate to the protein interior and quench the phosphorescence of deeply buried chromophores (14, 15). These parameters have been instrumental in uncovering subtle changes in the globular fold of enzymes brought about by the binding of substrates and allosteric effectors (16–19) or by variations in the physico-chemical conditions (20–22). In this paper, the phosphorescence properties of wild-type dI and two mutants, dI.Q132N and dI.R127M, are presented. It emerges that Trp-72 has an exceptionally well resolved phosphorescence spectrum and a slow phosphorescence decay in fluid solution, indicating that its local environment in dI.1 is conformationally homogeneous and unusually rigid. It is suggested that this feature is significant for the mechanism of action of transhydrogenase. Domain dI.1 is thought to close against dI.2 to control the relative movement of the dihydronicotinamide ring of NADH and the nicotinamide ring of NADP⁺ prior to hydride transfer.

EXPERIMENTAL PROCEDURES

All chemicals were of the highest purity grade available from commercial sources. Glycerol for fluorescence microscopy was purchased from Merck (Darmstadt, Germany). NADH, NAD⁺, NADPH, and NADP⁺ were obtained from Sigma. Acrylamide (>99.9% electrophoretic purity) was obtained from Bio-Rad. For phosphorescence measurements, water, doubly distilled over quartz, was purified by the Milli-Q Plus system (Millipore Corp., Bedford, MA). All glassware used for sample preparation was conditioned in advance by standing for 24 h in 10% HCl Suprapur (Merck, Darmstadt, Germany).

Recombinant dI and dIII from *R. rubrum* transhydrogenase were expressed in *Escherichia coli* and purified by column chromatography as described (8, 9). The dI protein was purified in 20 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol on Q-Sepharose Fast Flow (Amersham Biosciences) and Butyl Toyopearl (Tosoh) columns, and the dIII component

in 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 4 μ M NADP⁺ on Q-Sepharose Fast Flow (Amersham Biosciences), Q-Sepharose High Performance (Amersham Biosciences), and Butyl Toyopearl (Tosoh) columns. The dI mutant protein, dI.Q132N, was isolated as in Ref. 23, and dI.R127M was isolated by similar procedures (G. I. van Boxel and J. B. Jackson, unpublished data). Ammonium sulfate-precipitated dI was extensively dialyzed against sodium phosphate (20 mM, pH 7.5) or HEPES (20 mM, pH 7.3) before use in phosphorescence experiments.

For phosphorescence measurements in fluid solutions, it is paramount to rid the samples of all O₂ traces. The samples were placed in 5 \times 5 mm-square quartz cuvettes especially designed to allow thorough removal of O₂ by the alternative application of moderate vacuum and inlet of ultra-pure N₂ (12). For measurements in low temperature glasses, the solvent was 60/40 (v/v) glycerol/buffer (20 mM sodium phosphate, pH 7.5), and the protein concentration was 7 μ M. Acrylamide-quenching experiments were carried out as described before (14).

Fluorescence and Phosphorescence Measurements—All luminescence measurements were conducted on home-made instrumentation. Briefly, for emission spectra, continuous excitation was provided by a Cernax xenon lamp (LX150UV, ILC Technology, Sunnyvale, CA) whose output was selected (6 nm bandpass) by a 0.23-m double-grating monochromator (SPEX Fluorolog 1680, Spex Industries, Edison, NJ) optimized for maximum stray-light rejection. The emission collected at 90° from the excitation was dispersed by a 0.25-m grating monochromator (H-25, Jobin-Yvon) set to a bandpass of 0.3 nm. A two-position light chopper intersects either the excitation beam only (fluorescence mode) or both excitation and emission beams in alternate fashion in such a way that only delayed emission gets through to the detector (phosphorescence mode). The photomultiplier (EMI 9635QB) current was fed to a low-noise current preamplifier (SR570, Stanford, Sunnyvale, CA) followed by a lock-in amplifier (ITHACO 393, Ithaca, NJ) operated at the chopper frequency. The output was digitized and stored by a multifunction board (PCI-20428W, Intelligent Instrumentation Inc., Tucson, AZ) utilizing visual Designer software (PCI-20901S Version 3.0, Intelligent Instrumentation Inc., Tucson, AZ). Spectra were acquired at a scan rate of 0.2 nm s⁻¹ and with a time constant (lock-in amplifier) of 125 ms.

For phosphorescence decays, pulsed excitation was provided by a frequency-doubled, Nd/Yag-pumped dye laser (Quanta Systems, Milan, Italy) (λ_{ex} = 292 nm) with a pulse duration of 5 ns and a typical energy per pulse of 0.5–1 mJ. The phosphorescence emitted at 90° from the excitation was selected by an interference filter (DTblau, Balzer, Milan, Italy) with a transmission window between 410–450 nm. A gating circuit that inverted the polarity of dynodes 1 and 3, for up to 1.5 ms after the laser pulse, protected the photomultiplier (Hamamatsu R928, Hamamatsu Photonics, Japan) from the intense fluorescence light pulse. In spectral measurements, the photocurrent signal was amplified, digitized, and multiple sweeps were averaged by the same computer-scope system. All phosphorescence decays were analyzed in terms of a sum of exponential components by a non-linear least-squares fitting algorithm (Global Unlimited, LFD, University of Illinois).

RESULTS

Phosphorescence Spectrum of Transhydrogenase dI—The characterization of dI phosphorescence was initiated by recording a spectrum at low temperatures in vitreous glasses. At low temperature, the spectrum of Trp generally displays a pronounced vibronic structure with a relatively well resolved 0,0 vibrational band. Although the wavelength of the 0,0 band ($\lambda_{0,0}$) is related to the polarity/polarizability of the indole environment (10), its bandwidth (BW, the width at half height) reports on the structural homogeneity of the site (11). The spectrum of Trp-72 in glycerol/phosphate buffer (20 mM, pH 7; 60/40, w/w) at 140 K is shown in Fig. 3. $\lambda_{0,0}$ is at 408.0 nm, which is blue shifted by 3–4 nm relative to that of Trp in non-polar solvents. This implies that the triplet-state energy of Trp-72 is raised, relative to that in a fully non-polar site, by polar interactions with the surrounding polypeptide. In addition, the spectrum is exceptionally well resolved, with the 3.25 nm BW of the 0,0 vibrational band being the smallest reported to date for any protein. By comparison the BW of Trp-48 in azurin (a residue with a similarly blue-shifted fluorescence spectrum) is 6.5 nm (24), and that of Trp free in the solvent is 9.6 nm. It was also found that between 295–280 nm, the spec-

FIG. 2. The local environment of Trp-72 in the dI component of *R. rubrum* transhydrogenase. The strands, β a, β d, and β e are shown as C_α traces in gray. Segments of the main chain of loop β a- α 1, helix α 11, and selected amino acid side chains are shown with O, N, and S atoms in black. The side chain of Trp-72 is also shown in black. The gray dotted lines indicate van der Waals interactions between the indole ring of Trp-72 and polar groups. The diagram was constructed with Molscript (33).

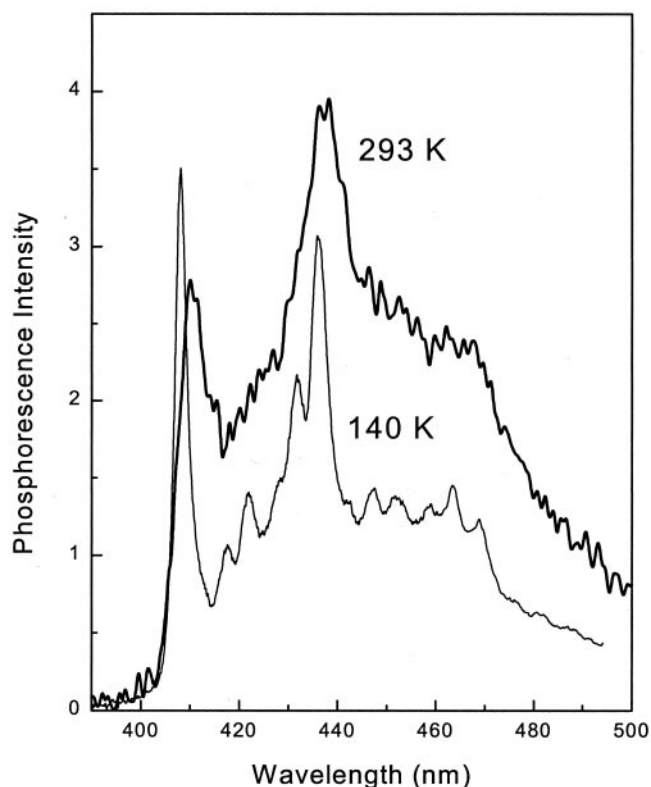
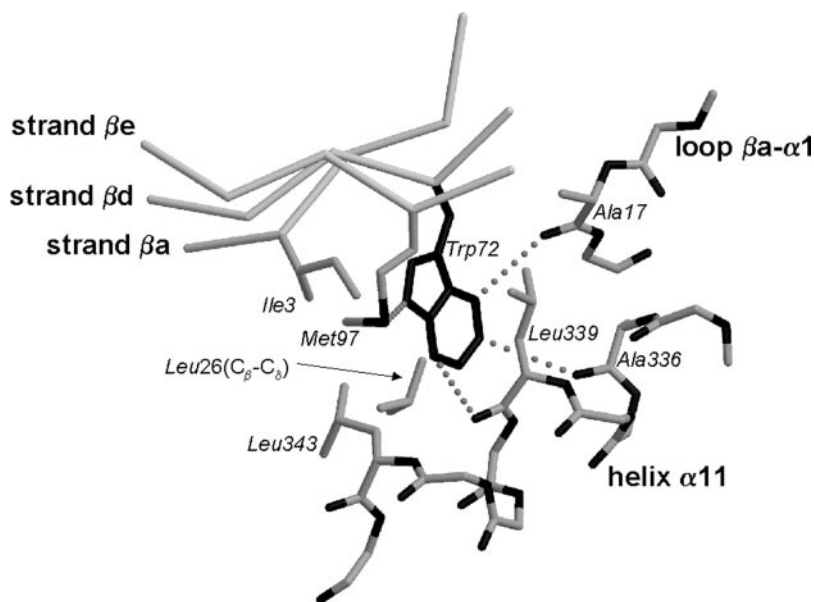


FIG. 3. The Trp phosphorescence spectra of dI in a glycerol/buffer (60/40, w/w) glass at 140 K (thin line) and in buffer at 20 °C (thick line). The buffer was 20 mM sodium phosphate, pH 7.5. The concentration of dI (in monomers) was 7 μ M in the glass and 1.2 μ M in the buffer. $\lambda_{\text{ex}} = 295$ nm.

trum is independent of the excitation wavelength. Indeed, after subtraction of the tyrosine component, which is identified by its characteristic contribution between 350–400 nm, the spectrum remained unaltered when the excitation wavelength was varied. Both the high resolution of the phosphorescence spectrum (a narrow distribution of excited-state energies) and the spectral invariance with respect to the excitation wavelength (a narrow distribution of ground-state energies) emphasize the structural homogeneity of the Trp-72 environment. The implications are that there is little conformational freedom in the

region of Trp-72 and that the structures of the two subunits in the dimer are equivalent.

Warming a low temperature glass into a fluid solution increases the flexibility of the structure of a protein. As a result of relaxation about the excited chromophore, the emission spectrum of the protein generally, therefore, undergoes a sizable red-shift and broadening. However, the phosphorescence spectrum of dI in buffer remains quite well resolved up to ambient temperature and red-shifts by only 2 nm relative to that seen in the rigid glass (Fig. 3). This is an unusually small shift when compared with the 5–10 nm observed for Trp residues in flexible or solvent-exposed sites (10). It indicates that spectral relaxation in dI is largely inhibited even on the long time scale of phosphorescence. This finding is consistent with the notion of a very rigid core around Trp-72 even at temperatures where the enzyme is fully functional.

Phosphorescence Decay of dI in Fluid Solutions—In fluid solutions at ambient temperature, both phosphorescence intensity and phosphorescence lifetime can be greatly reduced through the enhancement of radiationless processes promoted by the mobility of the protein/solvent matrix about the triplet probe. As a result, long-lived phosphorescence ($\tau \sim 0.1$ –1 s) from proteins in aqueous solutions at ambient temperature is owed exclusively to Trp residues that are buried in rigid sites. The lifetime of these Trp residues, when not influenced by intramolecular quenching reactions with proximal cysteine, cystine, histidine, or tyrosine side chains, is directly correlated to the local flexibility of the polypeptide (12, 13).

The phosphorescence emission of dI in buffer (20 mM Hepes, pH 7.3) is intense and exceptionally long lived. The decay at 20 °C is slow and almost uniform, with over 95% of the intensity having a lifetime (τ) of 2.9 ± 0.1 s (Fig. 4). This τ is more than half the value observed in low temperature rigid glasses (5.3 s), where the flexibility of the polypeptide is completely blocked. The τ for dI is the longest reported to date for any protein in fluid aqueous solutions. According to the correlation between τ and solvent viscosity (η) obtained with model compounds (12), the site of Trp-72 is very rigid and almost glass-like ($\eta_{\text{local}} \sim 3 \times 10^6$ cPoise). Because dI appears to form a very tight dimeric structure, the rate of interconversion with the monomer is likely to be slow during the phosphorescence lifetime. In this case, the homogeneous decay would imply identical lifetimes in each dI subunit of the dimer and indicate that the two cores of the dI.1 domains are structurally equivalent. A

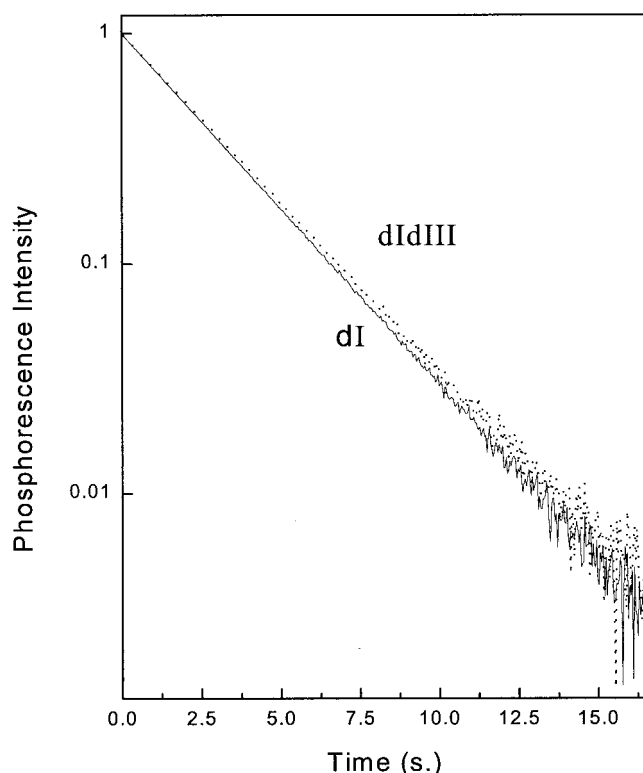


FIG. 4. Phosphorescence decay of dI and the $dIdIII_1$ complex in buffer (20 mM Hepes, pH 7.3) at 20 °C. Thick line, dI; dotted line, $dIdIII_1$. The dI concentration was 1.2 μ M (in monomers). $\lambda_{ex} = 292$ nm.

small fraction of the dI phosphorescence emission, 2–5%, has a lifetime of 0.9 s. This component was common to every sample, and its amplitude was insensitive to changes in temperature or buffer. It could represent the emission from a non-native fraction of dI or a protein impurity.

Temperature Dependence of the Phosphorescence Decay of dI—To determine the activation barrier of the structural fluctuations responsible for the triplet-state relaxation, the phosphorescence lifetime of dI in buffer was monitored across the 0–50 °C-temperature range. The value of τ decreased 3.8-fold, from 3.35 s at 1 °C to 0.89 s at 50 °C, whereas the decay remained homogeneous ($\alpha > 0.95$) throughout. At temperatures a few degrees above 50 °C, the phosphorescence became rapidly quenched, and the τ dropped to the sub-millisecond range, consistent with extensive unfolding of the globular structure. Under the conditions of the experiment, the process was irreversible in that the phosphorescence was not restored upon re-lowering the temperature of the sample.

The empirical relationship obtained between τ and solvent η with model compounds (12) was used to obtain a parameter ($1/\eta_{prot}$) that is an indirect measure of the rate of segmental motions about Trp-72 that are responsible for the τ reduction. The plot of $\ln(1/\eta_{prot})$ versus $1/T$ (formally equivalent to an Arrhenius plot) is linear between 10–50 °C (Fig. 5), and the slope yields an activation enthalpy of 23.3 kcal/mol. Activation energies above 20 kcal/mol are characteristic of tight protein cores formed by β -structures, sheets, and barrels, as found in proteins like azurin, alcohol dehydrogenase, and alkaline phosphatase (15). The temperature dependence of τ confirms that below 50 °C, the dI.1 domain retains a compact fold in which structural fluctuations are effectively blocked by activation barriers to unfolding. Note that the slope of the plot decreases below 10 °C. The deviation could indicate either a shift to lower-energy τ relaxation modes or an increasing insensitivity

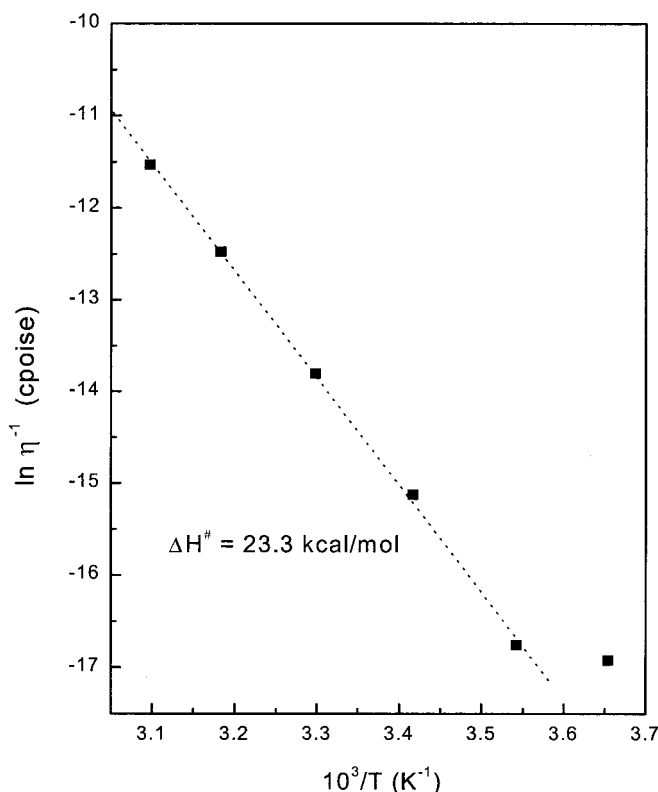


FIG. 5. Arrhenius plot of the rate of structural fluctuations ($1/\eta$) of dI in the region of Trp-72. The effective local viscosity η is derived from the empirical correlation between the intrinsic lifetime (τ_0) and solvent viscosity (12).

of τ to local fluidity as the lifetime approaches its upper limit of 5–6 s in rigid matrices.

Acrylamide Quenching of dI Phosphorescence—An independent assessment of the flexibility of the macromolecule in the region of Trp-72 can be obtained by observing how neutral molecules like acrylamide diffuse through the globular fold to the chromophore and quench its phosphorescence by a relatively short-range reaction (14). The long τ of dI permits, in principle, the determination of even very low bimolecular quenching rate constants. The phosphorescence decay is mono-exponential for acrylamide concentrations between 0–200 mM, and the lifetime decrease yields a linear Stern-Vollmer plot ($1/\tau = 1/\tau_0 + k_q[\text{acrylamide}]$) (Fig. 6). At 20 °C, the slope of the plot gives a second-order rate constant $k_q = 34 \pm 2 \text{ M}^{-1} \text{ s}^{-1}$. When compared with k_q values of $10^9 \text{ M}^{-1} \text{ s}^{-1}$ for solvent-exposed residues (14, 25), it is evident that the rate of migration of acrylamide to the site of Trp-72 is greatly reduced. Similarly small values of k_q were found for the compact regions of azurin and asparaginase (14). They are in accord with the long, phosphorescence decay and are consistent with the view that structural fluctuations in the core of dI.1 are greatly hindered. Further, the fact that the decay remains homogeneous despite acrylamide quenching indicates that the rate is similar for the two subunits of the dimer, suggesting again that their Trp-72 environments are equivalent. In contrast to the slow acrylamide migration inferred from phosphorescence experiments, data on the quenching of Trp-72 fluorescence yielded a Stern-Vollmer constant of $\sim 2 \text{ M}^{-1}$, which translates into a k_q value of $3.8 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ (9). Differences in quenching rates of several orders of magnitude between fluorescence and phosphorescence have been observed with several proteins and are attributed to efficient long-range interactions between the fluorescence state and the quencher in the solvent (14). This makes acrylamide an unsuitable quencher of Trp fluores-

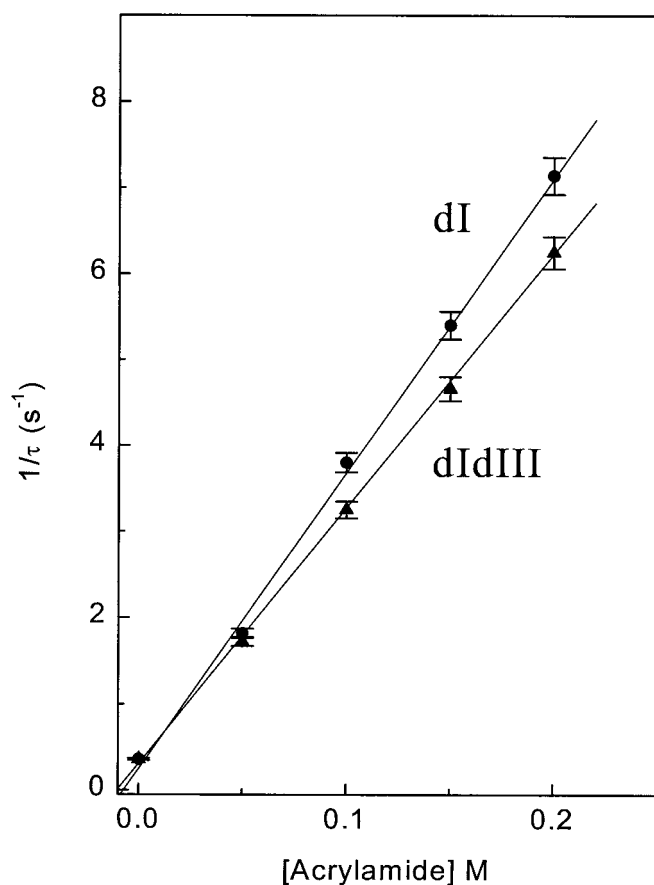


FIG. 6. Lifetime Stern-Vollmer plots for the acrylamide quenching of wild-type dI (●) and dI₂dIII₁ complex (▲) in 20 mM Hepes, pH 7.3. The dI concentration (in monomers) was 1.2 μ M and the dIII concentration was 0.9 μ M.

cence in accessibility studies on most proteins, including dI. The k_q value of 34 $\text{M}^{-1} \text{s}^{-1}$ found from the current phosphorescence measurements more realistically represents collisional quenching of Trp-72 by acrylamide diffusing through the protein matrix, and this low value is consistent with the very long triplet lifetime. In contrast to acrylamide, potassium iodide, an effective quencher of fluorescence from exposed Trp residues, had very little effect on the emission from Trp-72 in dI (9).

Effects of dI₂dIII₁ Heterotrimer Formation on the Phosphorescence of Trp-72—Mixtures of recombinant dI and dIII from *R. rubrum* transhydrogenase form a stable, dI₂dIII₁ heterotrimer: a second dIII binds to the complex with only very low affinity (26). This asymmetry is very interesting in the context of the mechanism of proton translocation by the enzyme, and we have, therefore, compared the phosphorescence of Trp-72 in isolated dI dimers (above) with that in dI₂dIII₁ complexes (Figs. 1 and 6 and Table I). The comparison is simplified by the fact that dIII is devoid of Trp residues. The experiments were performed with dIII in its NADP⁺ form. The results show that both spectral and lifetime characteristics of Trp-72 are unaffected when the dI dimer binds dIII. The only significant change is a small but reproducible reduction of the acrylamide quenching constant, indicating a decreased accessibility of the quencher to Trp-72 in the complex. The rate constants for dissociation of dI₂dIII₁ (into dI dimer and dIII monomer) and re-association are $\sim 50 \text{ s}^{-1}$ and $10^9 \text{ M}^{-1} \text{s}^{-1}$, respectively, and thus, at the protein concentrations used in the experiments, the complex is reformed many times during the phosphorescence decay. As a consequence of rapid dimer-trimer interchange, any potential asymmetry in phosphorescence lifetime

TABLE I
Spectral ($\lambda_{0,0}$ and BW), lifetime (τ_0), and acrylamide quenching (k_q) parameters characterizing the phosphorescence emission of W72 in WT and dI mutants and the effects of complex formation with dIII and coenzymes ([coenzyme] = 160 μ M)

Sample	$\lambda_{0,0}$ nm	BW nm	τ_0 s	k_q $\text{M}^{-1} \text{s}^{-1}$
Wild type				
dI	408.05	3.25	2.85 ± 0.1	34 ± 1.0
dI-dIII	408.00	3.30	2.95 ± 0.1	30 ± 1.2
dI-NADH	408.32	3.50	2.75 ± 0.2	
dI-dIII-NADPH	408.28	3.60	2.66 ± 0.2	
Q132N				
dI	408.20	3.13	2.95 ± 0.1	30 ± 0.9
dI-dIII	408.22	3.09	2.95 ± 0.1	25 ± 1.1
dI-NADH	408.34	3.60	2.80 ± 0.1	
dI-dIII-NADPH	408.24	3.50	2.61 ± 0.1	
R127M				
dI	408.17	3.22	2.80 ± 0.1	22.5 ± 1.1
dI-dIII	408.19	3.27	2.75 ± 0.1	19 ± 1.0
dI-NADH	408.20	3.70	2.50 ± 0.1	
dI-dIII-NADPH	408.12	3.22	2.41 ± 0.1	

between the dI subunits in the trimer would be masked. However, the observation that the phosphorescence lifetime of the complex is practically identical to that of the dI dimer, and the fact that there is no change in BW of the heterotrimer spectrum, strongly indicate that the protein conformation in the region of Trp-72 is identical in the two subunits and is not affected by dIII binding.

Effects of NADH Binding on the Phosphorescence of dI—dI binds NADH with a moderately high affinity ($K_d = 30 \mu\text{M}$). Binding of NADH to dI causes a small, 0.3 nm red shift of the spectrum, which is accompanied by a slight increase in BW from 3.25 to 3.50 nm (Table I). Hence, the binding of coenzyme has only minor effects on the local structure of dI.1, increasing slightly the exceptionally narrow spread in conformations of the dimer. The phosphorescence decay in buffer, at 20 °C, was measured at NADH concentrations up to 165 μM , which corresponds to 85% saturation of the binding sites ($K_d = 30 \mu\text{M}$, 5 μM dI). At the largest concentration, we find only a modest reduction of τ . The magnitude of the change depended on the coenzyme stock, indicating that quenching impurities are responsible for this decrease in lifetime. Note that, in principle, binding of the coenzymes NAD(P)H and NAD(P)⁺ to the protein can influence τ in two distinct ways: (i) by a structural rearrangement of the protein in the region of Trp-72, and (ii) by a through-space quenching interaction caused by energy transfer in the case of the reduced form and electron transfer in the case of the oxidized form. For through-space quenching to be effective, the nicotinamide moiety must be within the interaction range of Trp-72 ($\sim 13 \text{ \AA}$ and $\sim 17 \text{ \AA}$ for the reduced and oxidized forms, respectively) (19). In the crystal structure, Trp-72 is $\sim 20 \text{ \AA}$ from the bound nucleotide and, therefore, no through-space quenching is expected. The lack of NADH effects on the phosphorescence decay indicates that coenzyme binding to dI does not alter the polypeptide conformation of the core of domain dI.1 in the region of Trp-72.

Effects of NADPH on the Heterotrimer Phosphorescence—NADP⁺ binds very tightly to isolated dIII and to dI₂dIII₁ complexes ($K_d < 10^{-9} \text{ M}$). In the presence of excess NADPH, bound NADP⁺ is displaced from the dIII component on a time scale of a few minutes (27). Table I shows that the effect of exchanging NADP⁺ for NADPH on the phosphorescence lifetime of Trp-72 in dI₂dIII₁ complexes is very small. As with the effect of NADH, it is probably attributable to impurity-quenching from less than perfectly pure stocks. The lack of static quenching by bound nucleotides is again consistent with the large separation between Trp-72 and the coenzyme binding

sites observed in the crystal structure. The invariance of τ strongly suggests that the structure of the internal region of dI.1 that houses the probe is unchanged by both dIII complexation and coenzyme binding to either dI or dIII in the heterotrimer.

Effects of Mutations Q132N and R127M on the Phosphorescence Lifetime of Trp-72—All experiments conducted on the wild-type protein were repeated with the mutants dI-R127M and dI-Q132N. The mutated residues are located in the RQD loop of domain dI.1. This loop lines a region of the cleft between dI.1 and dI.2, and it forms a part of the hydride-transfer site. Although the two amino acid substitutions are located in the same region of dI, they have entirely different effects on catalysis. Thus, dI-R127M has a greatly decreased affinity for NADH (the K_d is raised >10-fold relative to wild-type dI, G. I. van Boxel and J. B. Jackson, unpublished results). In contrast, dI-Q132N has wild-type affinity for NADH but, in complex with dIII, it shows a greatly decreased hydride-transfer rate between bound nucleotides (k_{cat} is lowered >500-fold; Ref. 23). Both mutant proteins are dimeric and bind tightly to dIII. Investigation of the phosphorescence properties of these two mutants should reveal whether the large changes in activity are reflected in changes in the Trp-72 microenvironment.

The results of phosphorescence experiments with these two mutants show that both spectral and lifetime data, summarized in Table I, are remarkably similar to those of the wild-type protein. This finding is quite unexpected, given the sensitivity of τ to even minor conformational changes. It would seem that, despite the substitution of catalytically important residues in the nearby loop, the structural core of dI.1 surrounding Trp-72 retains its exceptional stability. Small differences in the acrylamide quenching constants were observed for dI-R127M and dI-Q132N (34 and 13%, respectively, of wild-type values), implying that the globular structure of dI.1 is slightly more compacted in the two mutant proteins. As with the wild-type dI, heterotrimer formation with dIII further decreases the accessibility of acrylamide to Trp-72.

DISCUSSION

During the last decade, there has been much progress in our understanding of the mechanism of action of transhydrogenase. Based on numerous mutagenesis studies, kinetic investigations and the availability of high-resolution structural information on dI and dIII, important details of the hydride-transfer step have been rationalized (1). However, because of inherent difficulties in the study of membrane-bound proteins, relative little information is available on the structure of dII and how proton translocation by means of this component is coupled to hydride transfer. The coupling mechanism is mediated by conformational changes within the protein, but our understanding of these changes is limited. By reporting on the nature and dynamic features of the local environment, Trp phosphorescence represents, to date, one of the most sensitive methods for the detection of conformational changes in proteins. Details of the local environment of Trp-72 are shown in Fig. 2 (6). The residue lies near the center of strand β d in the mainly parallel, twisted β -sheet of domain dI.1. Its side chain packs between those of two leucine residues and a methionine. The oxygen atoms of the main chain carbonyl groups of Ala-17, Ala-336, Leu-339, and the sulfur atom of Met-97 are located 3.6–4 Å from the Trp-72 indole. It may be noted that the tryptophan is not conserved in other species, but the equivalent residues (and the two previous residues) are always hydrophobic. There is a strongly conserved, positively charged amino acid residue following Trp-72, but its side chain points toward the other side of the β -sheet. Here, we report that both static and dynamic features of this structural core lead to the conclu-

sion that the region is exceptionally homogeneous and rigid. Notably, the rigidity is barely affected upon formation of the homodimer, the heterotrimer with dIII, the complexes with coenzyme NAD(P)H, and by mutations in the active-site loop emanating from dI.1.

A remarkably homogeneous polypeptide conformation about Trp-72 is inferred from spectral invariance with respect to the excitation wavelength (no ground-state heterogeneity), a narrow distribution in spectral energies (BW), a uniquely long phosphorescence lifetime, and a very low rate constant for acrylamide quenching. The phosphorescence and fluorescence spectral energies of a Trp residue in a protein are determined by the sum of dipolar (dipole-dipole and charge-dipole) and hydrophobic (dipole-induced dipole) interactions between the indole ring and the surrounding groups. Therefore, they provide information on the local polarity. Dipolar interactions can either raise or lower the energy of the excited state, depending on the orientation of charges or dipoles relative to the dipole of the indole ring. Hence, when the geometry is not unique, as in the case of multiple local peptide configurations, these interactions lead to considerable spectral broadening. Hydrophobic interactions, on the other hand, are generally weaker, depend on the polarizability of the neighboring groups, and are always stabilizing, independently of the geometry. A phosphorescence $\lambda_{0,0}$ of 408 nm is 3–4 nm blue-shifted relative to a fully non-polar site (10) and attests to a partial destabilization of the triplet state by dipolar interactions, presumably with the carbonyl groups of nearby Ala-17, Ala-336, and Leu-339 or the positive charge on the opposite side of the β -sheet whose influence can propagate over large distances in the low dielectric protein medium. In the presence of dipolar interactions, a narrow distribution of spectral energies (BW) is particularly significant because the lack of spectral broadening implies an essentially unique local protein configuration. In this context, it is pertinent to mention the very blue-shifted fluorescence spectrum of Trp-72 (λ^{max} is 310 nm) (9). The customary interpretation of a blue-shifted Trp fluorescence is that it indicates an apolar microenvironment. However, it has been pointed out that λ^{max} is most extensively blue-shifted for Trp in rigid polar solvents/protein sites, where relaxation (a spectral red shift) of the local polypeptide structure is blocked in the nanosecond time scale (10). Homogeneity in protein conformation in the region of Trp-72 is also inferred from the very uniform ($\alpha > 0.95$) phosphorescence (this work) and fluorescence (9) decays. Monoexponential fluorescence in single Trp proteins is rarely observed and is taken to indicate that the Trp side-chain adopts only one rotameric configuration. Similarly, the phosphorescence decay of individual Trp residues is often non-exponential, multiple lifetimes emphasizing that, at ambient temperature, various native-like states of the macromolecule, differing in internal flexibility, are in thermal equilibrium (28).

A τ of 2.9 s is the largest lifetime ever reported for a protein in buffer at 20 °C and translates into a local effective viscosity of 3×10^6 cP, suggesting a glass-like structure for the region. Evidently, relatively large activation barriers exceeding 20 kcal/mol are indicative of highly hindered motions of nearby side chains and backbone, a finding consistent with crystallographic B-factors having low values in the region of Trp-72 (2). Likewise, the minimal red shift of the phosphorescence spectrum in passing from a glass matrix at low temperature to fluid buffer at ambient temperature confirms that structural relaxation about the excited triplet excited state is slow even in the time scale of seconds. A compact protein matrix around Trp-72 is independently confirmed by the greatly hindered rate of acrylamide migration through the globular fold, as evidenced by the fact that k_q is 10^7 -fold less than is commonly observed

for surface Trp residues in proteins. Taken together, this information strongly suggests that dI.1 contains an extremely closely packed and homogeneous protein core. The unique properties of this core could make it a paradigm for the design of compact and conformationally homogeneous *de novo* proteins.

The phosphorescence lifetime of Trp is one of the most sensitive probes available for the detection of conformational changes in proteins. Subtle changes in the intramolecular quenching configuration, changes in accessibility to external quenchers, and changes in local flexibility will all affect the triplet lifetime (20, 28, 29). The large window of lifetimes, from sub-milliseconds to seconds, makes detection of conformational changes straightforward and offers very high sensitivity, particularly for buried Trp residues that exhibit a long τ . In numerous studies, measurements of τ have revealed subtle variations in the structure of proteins that could not be detected by other means (17–19, 30). Furthermore, wherever parallel measurements have been performed, changes in the protein structure detected by other techniques have always been reflected by a substantial alteration of τ . Thus, the measurements described in this report show that the dI component of transhydrogenase is exceptional in this respect. According to the phosphorescence data, the very rigid core of dI.1 does not seem to undergo conformational changes upon dI dimerization, nucleotide binding, or dIII complexation. This finding is surprising because (i) the two dI polypeptides in the crystal structure of the dI₂-dIII₁ complex have slightly different conformations, and (ii) NMR experiments show that there is a significant change in the conformation of a mobile loop at the active site of isolated dI upon nucleotide binding (31). Note also that one NAD(H) nucleotide is bound with high affinity by the heterotrimer, suggesting that the protein is asymmetric in solution as well as in the crystalline state (32). To our knowledge, this is the first example in which a rigid core around a Trp residue has been shown to prevent the transduction of a conformational change in a protein to the local environment of the residue. The only detected changes in the behavior of the Trp-72 phosphorescence were in the dI-Q132N and dI-Q127M mutants, where the k_q values for acrylamide quenching were lowered by 13 and 34%, respectively, relative to wild-type dI. These results suggest that the two mutations, both located in dI.1 where the component forms an interface with dIII, have reduced accessibility of acrylamide toward Trp-72.

The rigidity of the protein core of dI might be functionally relevant during turnover of the intact enzyme. Our working model for the mechanism of action of transhydrogenase derives from kinetic and thermodynamic data analyzed in the context of available crystal and NMR structures. Importantly, it was noted from the crystal structure of isolated dI that relative movement of dI.1 toward dI.2 leads to expulsion of the nicotinamide ring of bound NAD⁺ toward the rim of the inter-domain cleft (2). When the structure of the dI₂-dIII₁ complex became available (6), it was evident that equivalent movements are required during turnover of the intact enzyme to push the dihydronicotinamide ring of NADH toward the nicotinamide ring of NADP⁺. This is essential to “gate” the hydride-transfer reaction during inter-conversion of the open and occluded states of the enzyme: the reactive dihydronicotinamide ring of NADH and nicotinamide ring of NADP⁺ must be kept apart in the open state to prevent a disastrous redox reaction in the absence of proton translocation, and the rings are driven into apposition only in the occluded state to allow hydride transfer. The relative movements of dI.1 and dI.2 are probably linked to conformational changes in the helix D/loop D region of dIII, and, in turn, these events are driven by proton translocation

through the membrane-spanning dII. We suggest that the rigidity of dI.1, detected by the phosphorescence measurements, is important in maintaining tight coupling between the movements of helix D/loop D and the NAD⁺ nicotinamide. The dIII component has extensive contact surface with dI.2 but only a few interactions with dI.1. However, these involve a number of highly conserved amino acid residues, including those in the RQD loop. This feature of dI.1 lines that part of the cleft which forms the binding pocket of the nicotinamide ring for NAD(H). It is separated from the core of the domain by the linking helix α 11, and this region also has a preponderance of conserved amino acid residues. Thus, the core of dI.1 forms a rigid back wall for the conformationally mobile hydride-transfer site. This combination of mechano-chemical properties is probably essential to the proper functioning of the site: movement of the rigid core of dI.1 relative to dI.2 permits the compression in the RQD loop and associated structures that is necessary to cause the conformational changes of the nucleotides and protein into the ground state for the redox reaction. In principle, this view could be tested by substituting amino acid residues in the core of dI.1 to loosen the packing. With the phosphorescence of Trp-72 serving as a probe for the rigidity of the core, we should expect softer structures to fail to compress the catalytic site adequately for efficient hydride transfer.

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